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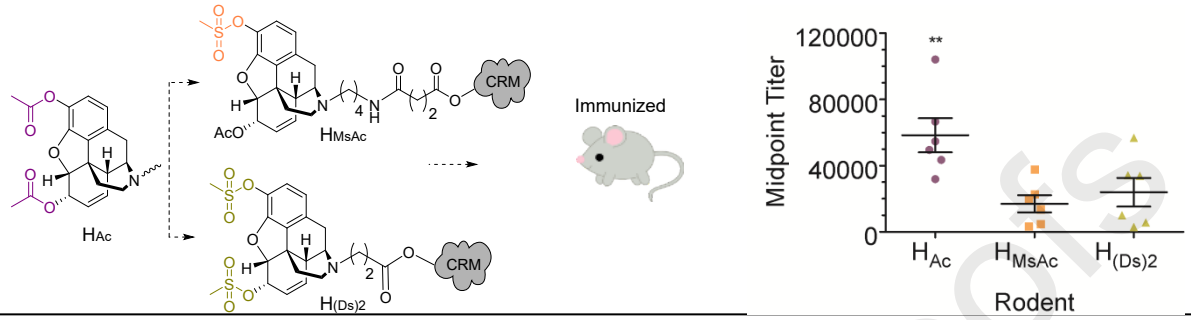
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Graphical Abstract

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Sulfonate-isosteric replacement examined within heroin-hapten vaccine design

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ABSTRACT

Heroin overdose and addiction remain significant health and economic burdens in the world today costing billions of dollars annually. Moreover, only limited pharmacotherapeutic options are available for treatment of heroin addiction. In our efforts to combat the public health threat posed by heroin addiction, we have developed vaccines against heroin. To expand upon our existing heroin-vaccine arsenal, we synthesized new aryl and alkyl sulfonate ester haptens; namely aryl-mono-sulfonate (H_{MsAc}) and Aryl/alkyl-*di*-sulfonate ($H_{(Ds)2}$) as carboxyl-isosteres of heroin then compared them to our model heroin-hapten (H_{Ac}) through vaccination studies. Heroin haptens were conjugated to the carrier protein CRM_{197} and the resulting CRM-immunoconjugates were used to vaccinate Swiss Webster mice following an established immunization protocol. Binding studies revealed that the highest affinity anti-heroin antibodies were generated by the H_{MsAc} vaccine followed by the H_{Ac} and $H_{(Ds)2}$ vaccines, respectively (H_{MsAc}> H_{Ac}>>H_{Ds2}. However, neither the H_{MsAc} nor $H_{(Ds)2}$ vaccines were able to generate high affinity antibodies to the psychoactive metabolite 6-acetyl morphine (6-AM), in comparison to the H_{Ac} vaccine. Blood brain bio-distribution studies supported these binding results with vaccine efficiency following the trend H_{Ac}>H_{MsAc}>>$H_{(Ds)2}$ The work described herein provides insight into the use of hapten-isosteric replacement in vaccine drug design.

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The abuse of heroin has been one major contributor to the current world opioid epidemic, incurring huge social and economic costs.[1, 2] Moreover, failure to solve the opioid problem has led to high rates of mortality, typically through overdose.[3, 4] Traditional pharmacotherapeutic treatments include opioid replacement therapy with μ-opioid receptor agonists, e.g., methodone and buprenorphine and the use of antagonists such as naltrexone. These medications have been successful in blunting cravings and withdrawal symptoms; however, these current treatments are still confounded by adverse side effects, including relapse and overdose.[5]

One emerging strategy to combat the opioid crisis is the use of immunopharmacotherapy wherein a drug like molecule (hapten) undergoes a series of vaccinations to train the immune system to mount an antibody response against the drug of choice that would otherwise evade immune surveillance.[6] Using this overall premise, a wide variety of antidrug vaccines have been investigated including cocaine[7], nicotine[8, 9], methamphetamine[10] and most recently opioids[11-23]. Common components for a successful antidrug vaccine include carrier protein (T-cell epitope), adjuvant and hapten.[24] We and others have invested great efforts in trying to create an effective heroin vaccine. However, crafting an efficacious heroin vaccine has been complicated by the need to target not only heroin but also its psychoactive metabolites, 6-acetyl morphine (6-AM) and morphine.[25-27]

In line with μ-opioid receptor binding and behavioral models examining heroin and its metabolites, it has become clear that antibody targeting of both heroin and its metabolite 6-AM is of utmost importance.[25] Thus, a successful heroin vaccine would entail the elicitation of antibodies capable of kinetically sequestering heroin and 6-AM in the peripheral circulation thereby reducing these entities from entering the brain.[25] Moreover, it is generally accepted that heroin undergoes rapid hydrolysis to 6-AM in serum by cholinesterases or by simple hydrolysis.[28, 29] Constructed upon these principles, ester hydrolysis of a heroin-hapten vaccine could greatly impact antibody specificity, affinity and ultimately a vaccines efficacy.[28] Herein, we disclose a new synthesis of heroin haptens obtained through isosteric replacement of the 3 and 6-carboxyl-esters with methanesulfonate esters. These new heroin-sulfonate hapten vaccines were tested for efficacy via drug-binding and blood/brain bio-distribution assays as part of an effort to find a more efficacious heroin vaccine.

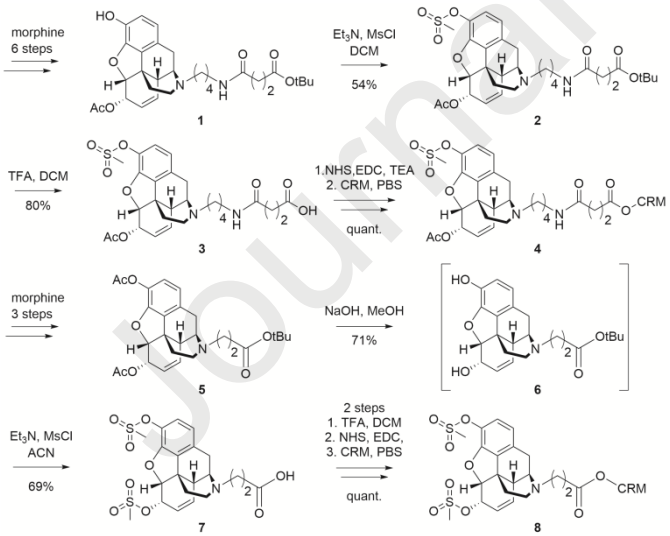
Our entry into ester isosteric replacement within heroin led us to look to the sulfonate moiety. We hypothesized that the sulfonate group would alter the immune response/recognition

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without significantly disrupting the immune recognition of heroin and 6-AM, our coveted targets. Using this logic, we choose two haptens for our study with additional variance found within the linker attachments. Thus, mono-sulfonate hapten (H_{MsAc}) was prepared with a "minimal" linker, and *di*-sulfonate hapten ($H_{(Ds)2}$) with an "extended" linker. Both (H_{MsAc}) and ($H_{(Ds)2}$) were compared to our "gold standard" heroin-hapten (H_{Ac}). The tactic used for the syntheses of the sulfonate-haptens was built upon a rich history of heroin-hapten design utilizing the nitrogen heteroatom for linker placement and carrier protein attachment.[22, 23] We note this overall motif has been widely scrutinized in past heroin-hapten iterations.[11] The key element of this approach was to install the sulfonate group at the late stage of the synthetic array to avoid potential nitrogen nucleophilic attack in the early steps, (Scheme 1). Starting from morphine sulfate, literature methods allowed for quick access to the extended linker alcohol **1**.[17] Mono-sulfonation of **1** gave **2** followed by deprotection under TFA conditions provided the first hapten **3**. Notably, the methanesulfonate group was stable under activation conditions of NHS/EDC and coupling to CRM carrier protein to give H_{MsAc} immunoconjugate **4** (Scheme 1).

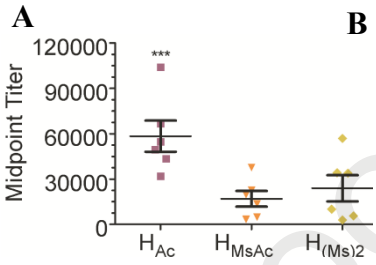
For the second isosteric hapten, $H_{(Ds)2}$ **7**, we elected to install a shorter linker allowing rapid access to **5,** thereby reducing the number of reaction steps from five to three (Scheme 1). Moreover, this also enabled for a significant quantity of diol **6** to be accessible as we found a large excess of mesyl chloride was needed for clean access to **7**. Deprotection of t-buytl (**6**) under the agency of TFA/DCM furnished the second hapten target **7** in quantitative yield. Similarly, this was then activated under NHS, EDC conditions, and then subjected to CRM relegation to give our final immunoconjugate **8.** Importantly, no difference in hapten coupling efficiency was observed. CRM-Immunoconjugates (**6** and **8**) were used for vaccination, whereas haptens **5** and **7** were coupled to the protein bovine serum albumin (BSA)[17] for ELISA plate coating. The hapten copy numbers for H_{Ac}, H_{MsAc}, and $H_{(Ds)2}$ were determined by MALDI-TOF MS.



Scheme 1. Synthesis and conjugation of sulfonate haptens.

The efficacy of H_{Ac}, H_{MsAc}, and $H_{(Ds)2}$ CRM-immunoconjugates was assessed by vaccination of Swiss webster mice with groups of n=6. Groups included a carrier protein negative control (KLH) and the three heroin hapten-CRM conjugates: H_{Ac}, H_{MsAc}, $H_{(Ds)2}$. Each group was administered vaccine on days 0, 14, and 28 with bleeds taken on days 21, and 42 (see SI). Success of active vaccination depends on the level of response, e.g. specificity and affinity of the antibodies generated. Therefore, midpoint titers were determined by ELISA. As shown in Figure 1 the H_{Ac} vaccine group presented us with greater titers than the H_{MsAc} and $H_{(Ds)2}$ groups.

To complement the ELISA data, SPR (surface plasma resonance spectroscopy) studies were conducted to examine antibody affinity and selectivity toward heroin and its psychoactive metabolite 6-AM. Consistent with our hypothesis, we observed large differences in affinity and immune response towards heroin for all the vaccines. In particular, the vaccine H_{MsAc} generated tighter binding affinity to heroin compared to the original H_{Ac} vaccine. (Figure 1). However, this improved affinity to heroin was not seen with $H_{(Ds)2}$ vaccine.



SPR IC_{50}, (nM)	6-AM	Heroin
H_{Ac}	21.1	493.4
H_{MsAc}	> 2μM	115.3
$H_{(Ds)2}$	>1000μM	>1000μM

Figure 1. Midpoint titers and binding affinity for the H_{Ac}, H_{MSAc} and $H_{(Ds)2}$vaccines. A) Midpoint titers at Week 6. Each column represents the average antibody titer in the sera of 6 replicate mice. Midpoint titers are reported as means ± standard error of the mean. Significance is denoted by an asterisk for midpoint titer from a one-way ANOVA and a Dunnetts post hoc test when comparing vaccinated groups to controls ***P ≤ 0.001; *P < 0.05 vs control. B) Calculated IC_{50} values (nM) determined by SPR analysis for each vaccine group (as pooled sera) at week 6 bleed.

Bio-distribution studies demonstrated that the H_{Ac} vaccine sequestered more active metabolite 6-AM and presented a lower brain to blood ratio compared to both H_{MsAc}, and $H_{(Ds)2}$ vaccine groups (Table 1). Interestingly, the quantity of 6-AM in brain samples for H_{MsAc} was lower than that for H_{Ac}. A result that agrees with the increased binding of heroin and a reduced quantity of 6-AM being sequestered. In stark contrast, the amount of 6-AM for $H_{(Ds)2}$ vaccine more closely resembled that of the KLH control. These observations complement other studies, highlighting the strict requirement for the 6-AM binding and recognition along the heroin metabolic pathway.[23]

Vaccine	Brain (ng/ml)	Blood (ng/ml)	Ratio
KLH	145	120	1.20
H_{Ac}	144	649	0.22
H_{MsAc}	102	239	0.43
$H_{(Ds)2}$	162	159	1.02

Table 1. Comparison of $H_{(Ds)2}$, H_{MsAc} and H_{Ac} vaccines on heroin vaccine efficacy in blood brain bio-distribution. Each vaccine group is comprised of pooled sera from 6 mice and control groups comprised of 4 mice. Blood and brain samples were analyzed for 6AM in ng/ml.

As stated, *vide supra*, heroin undergoes rapid hydrolysis by pseudocholinesterases that act to catalyze hydrolysis of heroin esters up to 10^{20} times relative to an uncatalyzed chemical processes.[28, 29] To rule out the possibility that hydrolysis of the sulfonate group embedded within our haptens could lead to alteration of binding to heroin/6AM and the immune response, we investigated aqueous hydrolysis of both H_{MsAc} and $H_{(Ds)2}$ haptens. Mass spectrometry experiments showed no evidence of hydrolytic degradation products for either sulfonate haptens over the course of a typical vaccination time period. This suggests that

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The data presented shows that incorporation of an aryl-sulfonate, (H_{MsAc}), contributes toward a favorable vaccine outcome, whereas double exchange for the esters within the heroin scaffold as observed with ($H_{(Ds)2}$) was deleterious to the vaccine's performance. The approach we undertook utilizing sulfonates as carboxylate isosteres while never tested in the hapten vaccine arena has been widely applied in medicinal chemistry to fine-tune the physicochemical properties of drugs, such as pKa, cell wall permeability and to a lesser extent, lipophilicity.[30] Moreover, structural alterations in the exchange of an ester for a sulfonate produce multiple different shape, electrostatic and hydrogen bonding interactions.[31, 32] On the whole we would suggest that both shape and electrostatics played a predominant role in the immune response seen and the results obtained for the H_{MsAc}, and $H_{(Ds)2}$ vaccines.

Opioid use disorder is expected to escalate over the coming years and become more of a burden to society. In an effort to expand our repertoire of heroin vaccines, we have developed new sulfonate-heroin haptens. The data presented shows an improvement in antibody affinity/specificity toward heroin with the H_{MsAc} vaccine, while double substitution as seen with the $H_{(Ds)2}$ hapten was not tolerated. Blood-brain biodistribution experiments largely corroborated binding data with sequestration of mainly heroin in the blood for the H_{MsAc} vaccine, which translated to a lower quantity of 6-AM present in the brain. While neither sulfonate heroin-hapten produced a more efficacious heroin vaccine then currently exists our findings do present a case to further investigate the use of isosteric replacement in haptens utilized in developing vaccines against drugs of abuse.

Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.

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